Determination of interstitial collagenase (MMP-1) in patients with rheumatoid arthritis

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Abstract

Objectives—To investigate whether interstitial collagenase (MMP-1) concentration in synovial fluid can be useful as a marker for disease activity in rheumatoid arthritis (RA), to determine the main route by which collagenase degrades the matrix of articular cartilage, and to investigate if an imbalance between metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMP) is responsible for the activity of MMPs in RA.

Methods—Collagenase concentrations were measured in synovial fluid and paired serum samples using a specific sandwich enzyme linked immunosorbent assay. Collagenase activities were also assayed in synovial fluid samples. Synovial tissues obtained from the same patient were examined by immunohistochemical staining and the numbers of cells expressing collagenase were counted.

Results—Collagenase concentrations in synovial fluid did not correlate with C reactive protein and collagenase levels in serum, but did correlate positively with the degree of synovial inflammation, and increased with increasing numbers of cells identified as expressing collagenase in synovial tissue. Collagenase activities did not correlate with TIMP-1 concentrations, but did correlate strongly with the ratios of collagenase concentration to TIMP-1 ($r = 0.73$).

Conclusion—The collagenase concentration in synovial fluid cannot be used as a marker for systemic disease activity, but can be used as a marker for the degree of synovial inflammation in the joint from which the sample is aspirated. In advanced RA, most of the collagenase is probably produced in synovial lining cells and released into synovial fluid, where it degrades the matrix of articular cartilage. An imbalance between MMP and TIMP may be of importance in the degradation of extracellular matrix of articular cartilage in RA.

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the joints. The matrix metalloproteinases (MMPs) are a family of enzymes that can degrade all components of the extracellular matrix.¹ Interstitial collagenase (E.C.3.4.24.7; MMP-1) is a member of the MMP gene family that cleaves the collagen triple helix to yield characteristic 1/4-3/4 products.²

Explants from human rheumatoid synovial tissue have been found to release large amounts of collagenase,³ and increased collagenase activity has been reported in rheumatoid synovial fluid.⁴ Collagenase was localised by immunostaining on the extracellular matrix components at the cartilage-pannus junction in RA,⁵ and immunohistochemical staining has subsequently shown it to be localised intracellularly in hyperplastic synovial lining cells of the rheumatoid synovium.⁶ However, it has also been reported that chondrocytes produce proteinases capable of degrading osteoarthritic and normal human cartilage,^{7,8} and that the matrix of cartilage was severely degraded when living cartilage was placed in contact with synovium.⁹

Collagenases in cell and tissue cultures are commonly synthesised and secreted as inactive, latent forms.¹⁰ Procollagenase can be activated in culture medium by trypsin,^{11,12} chaotrophic agents,^{13,14} and mercurial compounds,¹⁵ and in vivo by stromelysin (MMP-3).¹⁶ Van Wart *et al* have proposed the 'cysteine switch' activation mechanism for members of the matrix metalloproteinase gene family,¹⁷ but the process by which activation is achieved in vivo remains unclear. Cawston *et al* purified a collagenase inhibitor synthesised by newborn rabbit bone in tissue culture¹⁸ that bound to purified collagenase with a 1:1 stoichiometry. The inhibitor, named TIMP (tissue inhibitor of metalloproteinase), interacted with the active form of collagenase to form a tight enzyme-inhibitor complex that differed from latent collagenase.¹⁹ Dean *et al* proposed that an imbalance of MMPs and TIMP contributed to the pathogenesis of osteoarthritis (OA).²⁰

There have been no reports of studies in which synovial fluid, serum, and synovium were obtained from the same RA patient and MMP contents of the samples measured. In this study we assayed collagenase concentrations in synovial fluid and serum to investigate whether those in synovial fluid could be useful as a marker for disease activity in RA. We also compared synovial fluid concentrations of collagenase with the numbers

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Rheumatoid arthritis (RA) is characterised by chronic inflammation, which leads to degradation of the extracellular matrix and distortion of the architecture and function of

of cells expressing collagenase in synovial tissue (demonstrated by immunohistochemical staining) in order to determine the main pathway taken by collagenase to degrade the matrix of articular cartilage in RA. In addition, we measured collagenase activities and concentrations of collagenase and TIMP to investigate if an imbalance between MMPs and TIMP is responsible for the activity of MMPs in RA.

Patients and methods

MONOCLONAL ANTIBODIES AGAINST INTERSTITIAL COLLAGENASE (MMP-1)

Mouse monoclonal antibodies (clone K5E1, K2F7, K4H11) against interstitial collagenase (MMP-1) purified from serum free culture medium of P12-4 fibrosarcoma were prepared by means of a modification of a method described previously.²¹ Purified procollagenase had an M_r value of 52 000. Analysis of the effect of monoclonal antibodies on collagenase by immunoblotting showed that the antibodies reacted specifically with collagenase that had identical M_r values.

PATIENT SELECTION AND PREPARATION OF SAMPLES

Synovial tissue, synovial fluid, and serum were obtained from patients with RA, all of whom met the American College of Rheumatology (formerly the American Rheumatism Association) criteria for the diagnosis of RA.²² Synovial tissue was obtained at the time of total knee joint replacement surgery, and synovial fluid was aspirated from the same joint. The synovial tissue was fixed in 4% para-formaldehyde dissolved in phosphate buffered saline solution (PBS) for two hours, and then wrapped in paraffin. Synovial fluid and serum samples were centrifuged to remove cells and debris, and stored frozen at -70°C until required for assay.

IMMUNOHISTOCHEMICAL STUDIES AND QUANTIFICATION OF POSITIVE CELLS

For the immunohistochemical studies, sections of synovial tissue 2 μm thick were mounted on slides. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide in methanol for 30 minutes, followed by rinsing with PBS for 15 minutes. Normal horse serum was applied for 15 minutes as a blocking agent. This was followed by rinsing with PBS and application of the primary antibodies. The next day, the sections were rinsed with PBS and biotinylated secondary antibodies applied for 30 minutes. Streptavidin peroxidase was applied for 30 minutes. The sections were then exposed to diaminobenzidine and dilute hydrogen peroxide for seven minutes. The reaction was stopped with tap water, and the sections dehydrated and mounted.

The numbers of cells shown to express collagenase were counted on at least 20 different microscopic fields and the numbers averaged and graded on the following semi-quantitative scale: grade 0 = absent; 1+ = 1–5

cells per high power field (hpf) ($\times 400$); 2+ = 6–15 cells/hpf; 3+ = >15 cells/hpf.

DOUBLE ANTIBODY SANDWICH ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR COLLAGENASE

Collagenase concentrations were assayed using a specific sandwich ELISA. Ninety six well microtitre plates were coated overnight at 4°C with 10 $\mu\text{g}/\text{ml}$ of K5E1 in carbonate buffered saline solution. The plates were rinsed in 0.05% Tween 20-PBS, and incubated for one hour at room temperature (15°C) with Block Ace^R (Dainippon Seiyaku Co Ltd) in PBS to block non-specific protein binding in the wells. (Block Ace^R is a blocking reagent that contains 1% milk protein and organic acid buffer.) The blocker was rinsed from the plates and standard recombinant procollagenase or samples added for two hours at room temperature. Synovial fluid samples were diluted 1:1000 for assay with Block Ace^R; serum samples were diluted 1:10. The plates were then rinsed three times and incubated with K2F7 for two hours at room temperature. The antibodies were rinsed from the plates, which were then incubated with streptavidin peroxidase for 30 minutes at room temperature before rinsed four times, followed by 10 minutes incubation with *o*-phenylenediamine. The reaction was stopped by addition of normal sulphuric acid, and the absorbance at 490 nm was measured spectrophotometrically.

The assays detected both procollagenase and activated collagenase binding TIMP with almost the same sensitivity. Free active collagenase was assayed to a slightly different degree of sensitivity, but a dose-response curve for free active collagenase was obtained. TIMP could not be assayed adequately.

ONE STEP SANDWICH ENZYME IMMUNOASSAY (EIA) FOR TIMP-1

TIMP-1 concentrations in synovial fluid were measured by means of sandwich EIA Kits purchased from Fuji Chemical Industries Ltd. The sandwich EIA quantitatively measures not only free TIMP but also TIMP complexed with collagenase.^{23–25}

INFLAMMATION SCORES

The degree of synovial inflammation was analysed on haematoxylin and eosin stained sections using a modification of a method described previously.²⁶ Inflammation scores were determined as the sum of three components: synovial lining thickness (0 = 1–2 cells; 1+ = 3–4 cells; 2+ = 5–6 cells; 3+ = >6 cells), lymphoid follicles (0 = 0–1 per low power field (lpf) ($\times 40$); 1+ = 2–3/lpf; 2+ = 4–6/lpf; 3+ = >6 /lpf), and interfollicular features (0 = complete fibrosis; 1+ = mononuclear cell infiltration $<33\%$ of lpf; 2+ = 33–67%; 3+ = $>67\%$). They were then classified into four groups according to the semiquantitative scores: 0 = class 0; 1–3 = 1+; 4–6 = class 2+; 7–9 = class 3+.

COLLAGENASE ACTIVITY ASSAYS

Collagenase activities were assayed using the method reported previously.²⁷ In this study, synovial fluids were assayed for collagenase activities after activation with L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) treated trypsin. Fluorescein isothiocyanate labelled collagen was used as substrate. One unit of collagenase activity was defined as the amount of enzyme degrading 1 µg of collagen per minute under the conditions used.

STATISTICAL ANALYSIS

Data were analysed by the non-parametric Mann-Whitney *U* test, Student's paired *t* test, and Pearson correlation coefficients. *P* values less than 0.05 were considered significant.

Results

PATIENTS AND SAMPLE PREPARATION

Synovial fluid and serum samples were obtained from 41 patients (37 women and four men; mean age 60 years (range 32 to 89 years)). Synovial tissues were taken from 35 of these patients. TIMP-1 concentrations were assayed in samples from 35 patients, and collagenase activities measured in samples from 23.

RELATIONSHIP BETWEEN COLLAGENASE CONCENTRATIONS IN PAIRED SYNOVIAL FLUID AND SERUM SAMPLES

We measured collagenase concentrations in paired synovial fluid and serum samples from 41 patients. Collagenase concentrations in synovial fluids (mean 2361 (SD 2628) ng/ml) were significantly greater than those in sera (25.6 (21.9) ng/ml) ($p < 0.0001$). No obvious relationship existed between the levels of collagenase in synovial fluid and serum ($r = 0.207$, $p = 0.187$), and we found no relationship between the concentrations in synovial fluid and those of C reactive protein in serum, or between collagenase concentration in synovial fluid and the ARA (1949) Stage & Class criteria for the progression of rheumatoid arthritis and the functional

Figure 1 Relationship between synovial fluid concentrations of collagenase and inflammation scores. * $p < 0.05$; ** $p < 0.005$.

capacity of patients with the disease (data not shown).²⁸

RELATIONSHIP BETWEEN SYNOVIAL FLUID CONCENTRATIONS OF COLLAGENASE AND INFLAMMATION SCORES

Figure 1 illustrates the relationship between the concentration of collagenase in synovial fluid and the inflammation score classes. Six patients were in class 3+, 11 were in class 2+, 12 were in class 1+, and six were in class 0. The concentrations of collagenase in synovial fluid from patients with class 3+ inflammation ranged from 1991 to 11712 ng/ml (mean (SD) 4954 (3723) ng/ml ($n = 6$)); the values for class 2+ patients ranged from 478 to 11158 ng/ml (3790 (3163) ng/ml ($n = 11$)); those of class 1+ patients were from 320 to 6398 ng/ml (2400 (2182) ng/ml ($n = 12$)); and those of class 0 patients ranged from 97 to 1820 ng/ml (953 (707) ng/ml ($n = 6$)). A positive correlation was observed between the degree of synovial inflammation and the collagenase concentration in synovial fluid.

RELATIONSHIP BETWEEN GRADE FOR POSITIVE CELL COUNT AND COLLAGENASE CONCENTRATIONS IN SYNOVIAL FLUID

Collagenase was immunolocalised in hyperplastic synovial lining cells in rheumatoid synovium. Figure 2 shows a haematoxylin and eosin stained section of synovial tissue

Figure 2 Haematoxylin and eosin stained section of rheumatoid synovial tissue (left), and immunolocalisation of collagenase in the same rheumatoid synovial tissue, showing positive cells in the hyperplastic synovial lining cells (right). Bar represents 100 µm.

Figure 3 Relationship between the density of positive cells demonstrated by immunohistochemical staining and collagenase concentrations in synovial fluid (SF). * $p < 0.01$; ** $p < 0.001$.

and a serial section from the same synovium analysed for expression of collagenase by immunohistochemical staining. Very little staining was found in chondrocytes and fibroblasts at the cartilage-pannus junction.

With our system of grading by the number of cells immunohistochemically positive for collagenase expression, nine patients were grade 3+, nine were grade 2+, five were grade 1+, and 11 were grade 0. Figure 3 shows the collagenase concentrations in synovial fluid taken from patients belonging to each grade; concentrations in patients from grade 3+ ranged from 1189 to 11712 ng/ml (mean (SD) 4430 (3197) ng/ml ($n = 9$)); in grade 2+ patients they were from 924 to 11158 ng/ml (4954 (3157) ng/ml ($n = 9$)); those in grade 1+ patients ranged from 1609 to 3451 ng/ml (2346 (754) ng/ml ($n = 5$)); and concentrations in grade 0 patients ranged from 96 to 2040 ng/ml (842 (712) ng/ml ($n = 11$)). Collagenase levels in synovial fluid tended to increase with increasing numbers of positive cells, and a positive correlation existed between the degree of collagenase expression in synovial tissue and the collagenase concentrations in synovial fluid.

RELATIONSHIP BETWEEN CONCENTRATIONS OF COLLAGENASE AND TIMP-1 IN SYNOVIAL FLUID

In 35 synovial fluid samples measured, no positive correlation was found between the concentrations of collagenase and TIMP-1 ($r = 0.155$, $p = 0.3744$).

RELATIONSHIP BETWEEN COLLAGENASE ACTIVITIES AND CONCENTRATIONS OF TIMP-1 IN SYNOVIAL FLUID

We found no obvious relationship between the collagenase activities and the concentrations of TIMP-1 in 23 synovial fluid samples measured ($r = 0.235$, $p = 0.2814$) (fig 4).

RELATIONSHIP BETWEEN COLLAGENASE ACTIVITIES AND THE RATIOS OF COLLAGENASE CONCENTRATION TO TIMP-1 IN SYNOVIAL FLUID
Figure 5 illustrates the relationship between collagenase activities and the ratios of the concentrations of collagenase and TIMP-1 in 21 synovial fluid samples. There was a strong

Figure 4 Relationship between collagenase activities and TIMP-1 concentrations in synovial fluid.

positive correlation between collagenase activity and these ratios ($r = 0.73$, $p = 0.0001$).

Discussion

This study has shown: (i) collagenase concentrations in synovial fluid did not correlate with C reactive protein or collagenase concentrations in serum; (ii) collagenase levels in synovial fluid correlated positively with the degree of synovial inflammation; (iii) collagenase levels in synovial fluid increased with increasing numbers of cells positive for the expression of collagenase as demonstrated by immunohistochemical staining; (iv) no positive correlation between collagenase and TIMP-1 concentrations; (v) collagenase activity did not correlate with TIMP-1 concentrations, but (vi) did correlate strongly with the ratios of the concentrations of collagenase and TIMP-1.

In this study we examined collagenase concentrations in paired synovial fluid and serum samples taken from RA patients. No obvious relationship existed between the levels of collagenase in synovial fluid and serum, and the concentrations in synovial fluid correlated with neither C reactive protein concentrations in the serum nor ARA Stage or Class of disease progression. These data imply that synovial fluid collagenase concentration cannot be used as a marker for systemic disease activity in RA.

Figure 5 Relationship between collagenase activities and the ratios of the concentration of collagenase to that of TIMP-1 in synovial fluid.

We also compared synovial fluid concentrations with the degree of synovial inflammation. Using *in situ* hybridisation, Firestein *et al* reported a positive correlation between the degree of synovial inflammation and expression of collagenase mRNA in synovial tissue.²⁹ In our study, concentrations of collagenase in synovial fluid correlated with the degree of synovial inflammation. These data complement previous reports that cytokines derived from inflammatory cells, such as interleukin-1 and tumour necrosis factor, can modulate the amount of collagenase synthesised.³⁰⁻³⁴ While synovial fluid concentration of collagenase cannot be used as a marker for systemic disease activity, it can be used as a marker for the degree of synovial inflammation in the joint from which the sample is aspirated.

Collagenase has been immunolocalised in hyperplastic lining cells in rheumatoid synovium using an immunohistochemical method,⁶ and *in situ* hybridisation studies have also demonstrated production of collagenase mRNA in rheumatoid synovial lining cells.^{29 35 36} We applied monospecific antibodies against collagenase to synovium, cartilage, and the cartilage-pannus junction in the RA joint and were able to immunolocalise collagenase in synovial lining cells; very little staining was found in chondrocytes and fibroblasts at the cartilage-pannus junction. Woolley *et al* reported immunolocalisation of collagenase in the extracellular matrix at the cartilage-pannus junction, but the cells producing collagenase could not be identified.⁵ We graded synovial tissue according to the number of cells shown by immunohistochemical staining to express collagenase, and compared the grades with the concentrations of collagenase found in the synovial fluid. The concentrations of collagenase in the synovial fluid tended to increase with increasing numbers of positively staining cells.

Three apparently distinct processes are involved in cartilage degradation: hypertrophic synovial cells produce MMPs and release them into synovial fluid, where the enzymes degrade the articular cartilage matrix; fibroblasts at the cartilage-pannus junction secrete MMPs and directly degrade the cartilage matrix; and chondrocytes produce and secrete MMPs and then digest their own matrix. Fell *et al* reported marked degradation of both proteoglycans and collagen of the matrix when living cartilage was placed in contact with synovium,⁹ and Sapolsky *et al* have shown that chondrocytes of normal adult cartilage produce proteinases that are capable of degrading proteoglycans.⁷ Ehrlich *et al* demonstrated a collagenolytic enzyme in osteoarthritic and normal human cartilage,⁸ and Shinmei *et al* have suggested that autocrine modulation of chondrocyte metabolism may be the cause of cartilage breakdown in OA.³⁷

In the present study, collagenase was immunolocalised in synovial lining cells, but very little staining was found in the chondrocytes of articular cartilage and in the fibroblasts at the cartilage-pannus junction. A relationship was demonstrated between the degree of

expression of collagenase in synovial tissue and the concentration of collagenase in synovial fluid. These data suggest that, as far as collagenase is concerned, synovial cells are more important in the breakdown of the cartilage matrix than are chondrocytes. It has also been suggested that collagenase participates in the destruction of the entire cartilage surface, not just that of a localised area such as the cartilage-pannus junction. Hasty *et al* studied rats with collagen induced arthritis and proposed that chondrocytes may play a direct part in the earliest stages of degradation of their own matrix.³⁸ In the present study, we obtained all samples at the time of total knee joint replacement surgery and thus cannot comment on the importance of chondrocytes in the initial stage of RA. Our findings do suggest that, in advanced RA, most of the collagenase is produced in synovial lining cells and released into synovial fluid, where it degrades the articular cartilage matrix.

Both active and latent collagenase have been shown to be present in rheumatoid synovial fluid,^{39 40} and an inhibitor of collagenase demonstrated in newborn rabbit bone, TIMP, has been shown to bind to activated collagenase.^{18 19} It has been proposed that an imbalance between MMPs such as collagenase and TIMP contributes to the pathogenesis of OA.²⁰ We measured collagenase activities and TIMP-1 concentrations in synovial fluid, using an assay that included activation with trypsin. It is known that collagenase inhibitors such as TIMP-1, TIMP-2, and α_2 -macroglobulin may be inactivated by trypsin treatment, and thus the results of our assay may be overestimates. However, little active collagenase has been detected in synovial fluid,⁴¹ and the assay is not capable of measuring collagenase activity without the addition of an activating agent such as trypsin. We found no positive correlation between collagenase and TIMP-1 concentrations. This result is consistent with that reported by Clark *et al*.⁴²

It has been reported that procollagenase is secreted as both a minor glycosylated form and a major polypeptide.⁴³ Both the 57 kDa and the 52 kDa procollagenase could be activated by trypsin, generating their respective 47 kDa and 42 kDa active enzyme forms.⁴³ In the present study, the extent to which procollagenase contributes to the total (latent + active) concentration of collagenase in synovial fluid could not be determined; even when the concentration of total collagenase was assayed, an accurate molarity could not be calculated. It has previously been reported that no appreciable amount of apparently active collagenase was present in synovial fluid, the majority of the collagenase being in the latent form.⁴¹ If this is so, the ratio of concentrations of collagenase and TIMP will correspond approximately with the ratio of the molarity between them. We found a strong positive correlation between collagenase activity and the collagenase:TIMP ratio ($r = 0.73$, $p = 0.0001$). These data support the concept that an imbalance between MMP and TIMP is responsible for the degradation of extracellular matrix which occurs in RA.

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